

## EMBO Member's Review

# The ILK/PINCH/parvin complex: the kinase is dead, long live the pseudokinase!

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**Dynamic interactions of cells with their environment regulate multiple aspects of tissue morphogenesis and function. Integrins are the major class of cell surface receptors that recognize and bind extracellular matrix proteins, resulting in the engagement and organization of the cytoskeleton as well as activation of signalling pathways to regulate cell behaviour and morphogenetic processes. The ternary complex of integrin-linked kinase (ILK), PINCH, and parvin (IPP complex), which was identified more than a decade ago, interacts with the cytoplasmic tail of  $\beta$  integrins and couples them to the actin cytoskeleton. In addition, ILK has been shown to act as a serine/threonine kinase and to directly activate several signalling pathways downstream of integrins. However, the kinase activity of ILK and the precise functions of the IPP complex have remained elusive and controversial. This review focuses on the recent advances made towards understanding the specialized roles this complex and its individual components have acquired during evolution.**

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## Introduction

The structural organization of embryos and organogenesis require individual cells to sense and process extracellular information and to respond to these cues by changing cellular programmes such as cell division, death, proliferation, migration, and cell shape (Lecuit and Lenne, 2007). The extracellular information consists of chemical and mechanical cues imparted from growth factors and the extracellular matrix (ECM). The major cell surface receptors that cells use to recognize and assemble the ECM are integrins. Integrins are  $\alpha/\beta$  heterodimers that can assemble in different

combinations to confer substrate and signalling specificity. Lower eukaryotes such as worms and flies possess only a few integrin subunits, whereas mammals express 18  $\alpha$  subunits and 8  $\beta$  subunits that dimerize in 24 different combinations with cell-type-specific expression patterns (Hynes, 2002; Humphries *et al*, 2006). Deletion of individual subunits in both lower and higher organisms confirmed an essential role for integrins in development and tissue morphogenesis by regulating cell attachment to the ECM, cell migration, cell survival, cell cycle progression, and by modulating differentiation pathways or the structure and composition of the ECM (Legate *et al*, 2009).

Integrins are composed of large extracellular domains and relatively small cytoplasmic tails and exist on the plasma membrane in both inactive and active conformations, which exhibit either low or high affinity for extracellular ligands, respectively (Luo *et al*, 2007; Moser *et al*, 2009b). For integrins to become activated (inside-out signalling) non-integrin-mediated signals lead to the recruitment and binding of adaptor proteins such as talin and kindlin to the cytoplasmic tails of  $\beta$  integrins (Garcia-Alvarez *et al*, 2003; Tadokoro *et al*, 2003; Montanez *et al*, 2008; Moser *et al*, 2008, 2009a; Anthis *et al*, 2009), resulting in integrin activation through conformational changes in the cytoplasmic, transmembrane and particularly in the extracellular domains. However, for integrins to generate high-affinity adhesion or to relay intracellular signals, clustering of several integrin heterodimers is necessary to increase avidity towards the ligand. After clustering, integrins assemble multiprotein cytoplasmic adhesion complexes termed focal adhesions (FA), which enable them to induce a vast array of intracellular changes (outside-in signalling) (Ginsberg *et al*, 2005; Legate *et al*, 2009). Thus, the response of the cell to integrin ligation depends not only on the type of integrin heterodimer but also on the molecular composition of the adhesion complex. The integrin-linked kinase (ILK)/PINCH/parvin (IPP) complex is a central constituent of at least  $\beta 1$  and  $\beta 3$  integrin containing adhesion sites, from where it regulates multiple cellular processes. This review will address the recent advances made towards understanding the function of the IPP complex and its individual components both *in vivo* and *in vitro*.

## Molecular architecture of the IPP complex and its components

ILK, which is ubiquitously expressed in mammalian tissues, is composed of three structurally distinct domains. The N-terminus consists of five ankyrin repeats followed by a pleckstrin homology (PH)-like domain and a C-terminal kinase-like domain. The ankyrin repeats mediate the interaction with PINCH, a family of LIM domain only containing

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proteins consisting of two members, PINCH-1, and PINCH-2. Both PINCH proteins contain five LIM domains, the first of which binds ILK (Tu *et al*, 1999, 2001; Chiswell *et al*, 2008). The PH domain of ILK has been shown to bind phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P<sub>3</sub>) (Delcommenne *et al*, 1998; Pasquali *et al*, 2007). The C-terminal kinase-like domain binds several adaptor proteins including the parvins that consist of three members; the ubiquitously expressed  $\alpha$ -parvin (also known as actopaxin or CH-ILKBP),  $\beta$ -parvin (also known as affixin), which is primarily expressed in heart and skeletal muscle, and  $\gamma$ -parvin, which is expressed in the haematopoietic system (Nikolopoulos and Turner, 2000; Olski *et al*, 2001; Tu *et al*, 2001; Yamaji *et al*, 2001; Chu *et al*, 2006). Parvins are characterized by an N-terminal polypeptide stretch followed by two calponin homology (CH) domains, of which the second mediates the interaction with ILK (Tu *et al*, 2001).

The significant sequence homology of the C-terminus of ILK to Ser/Thr protein kinases was the reason why at the time of discovery the integrin tail-binding protein was called a kinase. However, ILK lacks well-conserved motifs required for eukaryotic protein kinase activity (Hanks *et al*, 1988), and the putative kinase activity and its physiological relevance has remained a subject of debate and controversy. Although ILK contains the lysine residue in subdomain II required for phosphotransfer and the A/SPE motif in subdomain VIII involved in substrate recognition, the GxGxxG consensus sequence of the kinase subdomain I required for covering and anchoring the non-transferable phosphates of ATP is not conserved in ILK from different species. This suggests that if ILK is indeed a kinase, this function would have evolved late during evolution. It is, however, even more difficult to reconcile that ILK lacks the catalytic base in subdomain VIb, which accepts the proton from the hydroxyl group of the substrate during the phosphotransfer reaction, as well as the DFG motif in subdomain VII required to align the  $\gamma$ -phosphate of ATP. A conserved lysine, which neutralizes the charge on the  $\gamma$ -phosphate of ATP, and a conserved asparagine, which chelates the secondary magnesium ions, both in subdomain VIb, are also missing (Figure 1). Owing to these characteristics, ILK has also been classified as a pseudokinase, a catalytically inactive remnant of an active kinase that uses its substrate recognition motif to interact with other proteins (Boudeau *et al*, 2006). It should be noted that in some cases proteins with a pseudokinase-like sequence have been shown to possess catalytic activity. Ca<sup>2+</sup>/calmodulin-

activated serine–threonine kinase (CAK), for example, binds ATP despite lacking two conserved residues that coordinate Mg<sup>2+</sup> ions in most kinases, but it functions as a Mg<sup>2+</sup>-independent kinase that is actually inhibited by Mg<sup>2+</sup> (Kannan and Taylor, 2008; Kornev and Taylor, 2009). Thus, the lack of key conserved residues has not been sufficient to exclude ILK as a kinase with an unusual catalytic mechanism.

## The kinase activity of ILK—the end of a controversy

As integrins themselves lack enzymatic activity, they propagate intracellular signals by recruiting signalling proteins such as tyrosine and serine/threonine kinases to their cytoplasmic tails. In the initial study identifying ILK as a direct binding partner of  $\beta$ 1 integrin, Dedhar and co-workers showed that bacterially expressed recombinant ILK possesses kinase activity and phosphorylates serine and threonine residues in the cytoplasmic tail of  $\beta$ 1 integrin (Hannigan *et al*, 1996). Since then, a large number of studies have been published on the potential kinase activity of ILK, claiming that ILK can directly phosphorylate a diverse set of substrates ranging from Akt, a kinase regulating key cellular functions such as cell cycle progression, survival, differentiation, and energy homeostasis, to myosin light chain (MLC) whose phosphorylation regulates actomyosin contractility and vascular tone (Legate *et al*, 2006).

Several lines of biochemical and cell biological evidence have supported the initial view that ILK might be an active kinase (Hannigan *et al*, 1996). It has been shown that recombinant ILK expressed in bacteria can phosphorylate the cytoplasmic tail of  $\beta$ 1 integrin as well as the model substrate myelin basic protein (Hannigan *et al*, 1996; Delcommenne *et al*, 1998). Furthermore, purified ILK from mammalian cell extracts was shown to co-immunoprecipitate and phosphorylate Akt (Persad *et al*, 2001). Mutational analysis has been used to gain further insight into the catalytic activity of ILK, and several mutations have been described to abrogate the kinase activity *in vitro*. A serine (S) to alanine (A) substitution in the potential autophosphorylation site (S343A), an arginine (R) to A substitution in the potential PtdIns(3,4,5)P<sub>3</sub>-binding site of the PH domain (R211A), or a lysine (K) to A or to methionine (M) substitution in the putative ATP-binding site (K220A/M) have all been shown to result in a catalytically inactive ILK (Persad *et al*, 2001; Filipenko *et al*, 2005), whereas an S to aspartate

Kinase subdomain:	ATP binding			Peptide binding and phosphotransfer				
	I	II	III	VIb	VII	VIII	IX	XI
Kinase consensus	G-G--G.....A-K.....E.....	DLK--N.....	DFG.....	APE.....	D-G.....	R		
<i>Drosophila</i> ILK	S- <b>T</b> -G.....V- <b>K</b> ..... <b>E</b> .....	<b>YHL</b> - <b>H</b> .....	<b>DAK</b> .....	<b>SPE</b> .....	<b>D</b> - <b>A</b> .....	<b>R</b>		
<i>M. musculus</i> ILK	N- <b>N</b> -G.....V- <b>K</b> ..... <b>E</b> .....	<b>HAL</b> - <b>R</b> .....	<b>DVK</b> .....	<b>APE</b> .....	<b>D</b> - <b>A</b> .....	<b>R</b>		

**Figure 1** The kinase homology domain of ILK lacks several critical residues present in eukaryotic serine/threonine kinases. Alignment of highly conserved amino acids within the 12 subdomains of eukaryotic serine/threonine kinases (Hanks *et al*, 1988) with *Drosophila* and *Mus musculus* ILK. Conserved amino acids are marked in bold, and their counterparts in the ILK sequences are shown in red. Conserved amino acids that are not present in the ILK sequences are highlighted with blue boxes. Most importantly, ILK lacks the catalytic base in subdomain VIb. Substitution of the invariant lysine in subdomain II (ATP binding) to either an alanine or methionine (K220A/M in *M. musculus* ILK; marked with \*) or of the invariant glutamate in subdomain VIII (substrate recognition) to a lysine (E359K in *M. musculus* ILK; marked with \*\*) have been shown to render ILK catalytically inactive.

(D) substitution in the autophosphorylation site (S343D) was shown to generate a hyperactive kinase (Persad *et al*, 2001). Importantly, however, these mutations have also been shown to disrupt the interaction of ILK with essential binding partners. The inactivating R211A mutation apparently disrupts the interaction with  $\alpha$ -parvin and impairs the recruitment of ILK to FAs (Attwell *et al*, 2003), whereas the K220A mutation reduces  $\beta$ -parvin binding (Yamaji *et al*, 2001). These findings together with the observation that a combination of two inactivating mutations (S343D and K220M) can reverse the kinase dead phenotype despite abolishing the ability to bind ATP (Lynch *et al*, 1999), suggested that these mutations might affect the activation status of downstream substrates such as Akt phosphorylation by an indirect mechanism.

A breakthrough came with genetic studies in *Caenorhabditis elegans* and *Drosophila melanogaster*, which failed to confirm the kinase function of ILK *in vivo*. The reported kinase dead ILK mutants were capable of fully rescuing the severe phenotypes caused by ILK deletion in both species, indicating that ILK functions as a scaffold protein and not as a kinase in invertebrates (Zervas *et al*, 2001; Mackinnon *et al*, 2002). Controversy still remained, as it was argued that ILK might have acquired catalytic activity later in evolution. Some genetic studies in mice failed to show a critical role for ILK in phosphorylating key substrates, despite showing overt phenotypes related to impaired actin organization; ILK-deficient fibroblasts, chondrocytes, or keratinocytes do not show changes in Akt or Gsk-3 $\beta$  phosphorylation (Grashoff *et al*, 2003; Sakai *et al*, 2003; Lorenz *et al*, 2007). In addition, depletion of ILK in vascular smooth muscle cells (vSMCs) results in hyperphosphorylation of the proposed substrate MLC (Kogata *et al*, 2009; Montanez *et al*, 2009). In contrast, ablation of ILK in the heart, skeletal muscle, nervous system, or macrophages abrogated phosphorylation of Ser473 of Akt (Troussard *et al*, 2003; White *et al*, 2006; Wang *et al*, 2008a; Pereira *et al*, 2009), raising the possibility of redundancy or tissue-specific kinase function.

Owing to this controversy, it was important to determine whether the catalytic activity of ILK might be specific for mammals or certain cell types, and whether phosphorylation of key substrates was normal in ILK-null mice and cells because of compensation by another integrin-associated kinase. We established knock-in mouse strains with point mutations in ILK that were reported to affect ILK kinase activity. Surprisingly, knockin mice carrying mutations in the putative PH domain (R211A) or in the autophosphorylation site (S343A or S343D) are completely normal and do not show changes in Akt or Gsk-3 $\beta$  phosphorylation or actin organization downstream of integrins (Lange *et al*, 2009). In contrast, mice carrying point mutations in the potential ATP-binding site (K220A/M) die shortly after birth because of kidney agenesis. This phenotype does not result from impaired kinase activity, as the mutations did not alter the phosphorylation levels of reported ILK substrates *in vivo*. In addition, no evidence of kinase activity was detected *in vitro* (Lange *et al*, 2009). However, these mutations selectively impair the interaction of ILK and  $\alpha$ -parvin. In line with this, similar kidney defects occur also in  $\alpha$ -parvin-null mice (Lange *et al*, 2009; Montanez *et al*, 2009). Interestingly, the K220M mutant can fully rescue the developmental defects of ILK-null *Drosophila* (Zervas *et al*, 2001), which is likely because of

major differences in organ systems of mammals and invertebrates. Flies and worms, for example, do not have kidneys.

Despite the lack of high-resolution structural data of the kinase-like domain as further proof for the pseudokinase function of ILK, it is now clear that the proposed kinase activity does not exist and, therefore, cannot have a role in mammalian development or adult life, or in the function of ILK as a mediator of the integrin-actin linkage in cells. This calls for the re-evaluation of a large number of studies published on the role of the kinase activity in numerous cellular processes. Several of the amino acid substitutions used in these studies proposed to abrogate the catalytic activity of ILK actually disrupt the function of the kinase homology domain as a critical and highly conserved mediator of protein-protein interactions at adhesion sites. Despite this conserved scaffold function it is also obvious that the complexity of mammalian tissue morphogenesis is facilitated by the assembly of more specialized adhesion complexes with distinct, tissue-specific signalling functions not present in lower organisms. This complexity is partly achieved by the different cellular functions of the PINCH and parvin isoforms, but also through interactions of ILK with various other structural and signalling proteins (Table I). Therefore, complete characterization of the ILK interactome as well as careful analyses of the functional significance of the various interactions represent important tasks for future research.

## Assembly of distinct IPP complexes

The assembly of the mammalian IPP complex occurs before adhesion, suggesting that it assembles in the cytoplasm and is subsequently recruited to integrin adhesions (Zhang *et al*, 2002). ILK was identified in yeast-two-hybrid experiments to directly bind to the cytoplasmic tail of  $\beta$ 1 integrin. Therefore, it was hypothesized that this direct interaction facilitates the recruitment of the IPP complex to FAs (Hannigan *et al*, 1996). However, a detailed molecular analysis of this interaction is still lacking, and the binding site on the integrin tail has not been mapped. Moreover, *Drosophila* ILK does not bind  $\beta$  integrin ( $\beta$ PS) indicating that a direct interaction is not required for its function in lower organisms (Zervas *et al*, 2001). Nevertheless, yeast-two-hybrid assays in the same study showed that *Drosophila* ILK can weakly bind human  $\beta$ 1 integrin, whereas human ILK does not bind  $\beta$ PS, suggesting that evolutionary changes in the cytoplasmic tail of  $\beta$ 1 integrin could have generated a binding site for ILK (Zervas *et al*, 2001). Recent studies, however, reported that the interaction between ILK and the integrin might be indirect also in mammals. Depletion of the FA protein kindlin-2, which directly binds to  $\beta$  integrin tails and also interacts with ILK, leads to the loss of ILK or PINCH from FAs, even when integrin activation and clustering was restored exogenously by the addition of manganese (Chen *et al*, 2008; Montanez *et al*, 2008). In addition, studies from our laboratory using quantitative proteomics to identify proteins that bind to the cytoplasmic tail of  $\beta$ 1 integrin have failed to detect ILK whereas kindlin-2 and talin are readily detected (Meves and Fässler, unpublished data). It is currently unclear, however, whether the kindlin-2-mediated recruitment of ILK to integrin tails occurs only in a subset of cells. Likewise, it has not been tested whether all kindlin family members can bind ILK. It has also been shown that a point mutation in the

**Table 1** The ILK interactome

Binding partner	Proposed function	Mode of interaction	References
$\beta$ 1 integrin	Anchorage-independent growth	Direct <sup>a</sup>	Hannigan <i>et al</i> (1996)
$\beta$ 3 integrin	Platelet aggregation	NA	Pasquet <i>et al</i> (2002)
PINCH-1/2	Stabilization of IPP complex, cell spreading, migration	Direct <sup>a,b,c</sup>	Chiswell <i>et al</i> (2008); Tu <i>et al</i> (2001); Tu <i>et al</i> (1999)
$\alpha$ / $\beta$ / $\gamma$ -Parvin	Stabilization of IPP complex, cell spreading, migration	Direct <sup>a,c</sup>	Nikolopoulos and Turner (2000); Tu <i>et al</i> (2001); Yamaji <i>et al</i> (2001)
Paxillin	Recruitment of ILK to focal adhesions	Direct <sup>c</sup>	Nikolopoulos and Turner (2001); Nikolopoulos and Turner (2002)
Kindlin-2	Recruitment of ILK to focal adhesions	NA	Mackinnon <i>et al</i> (2002); Montanez <i>et al</i> (2008)
Thymosin- $\beta$ 4	Actin polymerization, Akt phosphorylation	Direct <sup>c</sup>	Bock-Marquette <i>et al</i> (2004); Fan <i>et al</i> (2009)
ILKAP	Regulation of Gsk-3 $\beta$ signalling	Direct <sup>a</sup>	Leung-Hagesteijn <i>et al</i> (2001)
Rictor	Akt phosphorylation, cell survival	Direct <sup>a</sup>	McDonald <i>et al</i> (2008b)
EphA1	Cell shape and motility	Direct <sup>a</sup>	Yamazaki <i>et al</i> (2009)
Akt1	Akt1 phosphorylation, cell survival	NA	Persad <i>et al</i> (2001)
ELMO-2	Cell polarity	NA	Ho <i>et al</i> (2009)
c-Src	Phosphorylation of cofilin, actin organization	NA	Kim <i>et al</i> (2008)
kAE1	Actin linkage and membrane stability of kAE1 in kidney	NA	Keskanokwong <i>et al</i> (2007)
CNKSR3 gene chromatin	Regulation of transcription	NA	Acconcia <i>et al</i> (2007)
RUVBL1	Spindle assembly	Indirect <sup>a</sup>	Fielding <i>et al</i> (2008)

Abbreviations: ILK, integrin-linked kinase; IPP, ILK/PINCH/parvin; ILKAP, ILK-associated phosphatase; ELMO-2, engulfment and cell motility 2; kAE1, kidney anion exchanger 1; NA, not analysed.

<sup>a</sup>Demonstrated with yeast-two-hybrid.

<sup>b</sup>Demonstrated with co-crystallization.

<sup>c</sup>Demonstrated with recombinant proteins.

kinase homology domain of ILK, which abolishes its interaction with the adaptor protein paxillin, prevents ILK localization to FAs (Nikolopoulos and Turner, 2001, 2002). However, paxillin binding seems not to be sufficient to recruit ILK, as paxillin localizes to integrin adhesions in the absence of kindlin-2, whereas ILK does not (Montanez *et al*, 2008). Therefore, it is likely that coordinated interplay between kindlin-2 and paxillin, instead of a direct interaction between ILK and the  $\beta$ 1 integrin tail, facilitates the localization of the IPP complex to integrin adhesions. It is of course possible that later during the course of adhesion maturation the interaction of certain proteins with integrin cytoplasmic tails become displaced by ILK. An alternative possibility to such a sequential binding mode is that direct interactions of ILK with integrins may be restricted to certain cell types. Detailed molecular mapping of the interaction sites between ILK and kindlin-2 as well as between ILK and  $\beta$ 1 integrin and analyses of the relevance of these interactions *in vivo* are required to obtain answers to these questions.

The stability of each individual component of the IPP complex depends on the assembly of the complex. Depletion of ILK or PINCH leads to a decrease, albeit not a total loss, in the protein levels of the other two complex members (Fukuda *et al*, 2003; Li *et al*, 2005). This has made it difficult to assess the independent functions of the IPP proteins. Interestingly, however, multiple cell types of both epithelial and mesenchymal lineages express both isoforms of PINCH as well as both  $\alpha$ - and  $\beta$ -parvin. Binding of PINCH and parvin isoforms to ILK is mutually exclusive (Fukuda *et al*, 2003; Montanez *et al*, 2009), allowing cells to engineer molecularly distinct IPP complexes that have specific functions in modulating integrin signalling. Forced overexpression of PINCH-2 in PINCH-1-depleted cells can restore the assembly of the IPP complex, but this complex is unable to

compensate for the functional defects caused by the loss of PINCH-1, whereas PINCH-1 can fully compensate for the loss of PINCH-2 both *in vivo* and *in vitro* (Braun *et al*, 2003; Fukuda *et al*, 2003; Li *et al*, 2005; Stanchi *et al*, 2005). Deletion of  $\alpha$ -parvin leads to an upregulation of  $\beta$ -parvin expression, which acts to stabilize ILK and PINCH-1 levels, resulting in successful recruitment of this particular IPP complex to FAs. This is, however, not sufficient to functionally compensate for the loss of  $\alpha$ -parvin (Montanez *et al*, 2009). Thus, a model of signalling specificities through molecularly distinct IPP complexes is beginning to emerge. This will be discussed in more detail later in this review.

### An update of the functions of the IPP complex *in vivo* and *in vitro*

The biological functions of the IPP complex proteins have been extensively studied in several organisms and cell types (Table II). Genetic ablation of ILK or PINCH-1 in mice results in embryonic lethality (Sakai *et al*, 2003; Li *et al*, 2005). Mice lacking ILK expression die during peri-implantation because of a failure in epiblast polarization, which is associated with severe defects in F-actin organization at adhesion sites (Sakai *et al*, 2003). ILK-deficient fibroblasts display defects in cell adhesion, spreading, and migration because of a delay in the formation of FAs that also fail to mature and are poorly linked to a disorganized actin cytoskeleton (Sakai *et al*, 2003; Stanchi *et al*, 2009). The defective maturation of ILK-deficient FAs into fibrillar adhesions leads to defects in deposition of the fibronectin matrix (Stanchi *et al*, 2009). Interestingly, this function requires the interaction of ILK with  $\alpha$ -parvin but not with PINCH-1 (Stanchi *et al*, 2009). The essential role of ILK in linking integrins to the actin cytoskeleton has been further confirmed in several tissue and cell types (McDonald *et al*,

**Table II** *In vivo* analyses of IPP proteins

Gene	Organism	Tissue	Phenotype	References	
ILK	<i>Caenorhabditis elegans</i> <i>Drosophila melanogaster</i>		Embryonic lethality; muscle attachment defect	Mackinnon <i>et al</i> (2002)	
			Embryonic lethality; actin detachment from muscle membrane, adult wing blisters	Zervas <i>et al</i> (2001)	
	<i>Xenopus laevis</i> <i>Zebrafish</i>		Embryonic lethality; blastopore closure and axis elongation defects	Yasunaga <i>et al</i> (2005)	
			Embryonic lethality; cardiovascular defects	Bendig <i>et al</i> (2006); Friedrich <i>et al</i> (2004); Postel <i>et al</i> (2008) Sakai <i>et al</i> (2003)	
	<i>Mus musculus</i>	Constitutive	Embryonic lethality between E5.5 and E6.5; abnormal epiblast polarization, impaired cavitation, cell detachment from the ECM	Grashoff <i>et al</i> (2003); Terpstra <i>et al</i> (2003)	
		Bone (chondrocytes; Col2-Cre)	Perinatal lethality; chondrodysplasia, dwarfism, and respiratory distress	White <i>et al</i> (2006)	
		Cardiovascular system (cardiomyocytes; Mck-Cre)	Cardiomyopathy and heart failure	Friedrich <i>et al</i> (2004)	
		Cardiovascular system (EC; Tie2-Cre)	Embryonic lethality between E10.5 and E12.5; embryonic and extra-embryonic vascular defects	Kogata <i>et al</i> (2009)	
		Cardiovascular system (vSMC; PDGFRβ-Cre)	Embryonic lethality between E13.5 and E18.5; abnormal vessel wall formation	Lorenz <i>et al</i> (2007); Nakrieko <i>et al</i> (2008)	
		Skin (keratinocytes; K5-Cre and K14-Cre)	Epidermal defects and hair loss	Chevraya <i>et al</i> (2007); Wang <i>et al</i> (2008a)	
		Skeletal muscle (HSA-Cre)	Mild progressive muscular dystrophy	Liu <i>et al</i> (2005)	
		Immune system (T cells; Lck-Cre)	T-cell chemotaxis and survival defects	Tucker <i>et al</i> (2008)	
Haematopoietic system (platelets; Mx1-Cre)		Abnormal platelet aggregation, granule secretion, and thrombus formation	Ckretsi <i>et al</i> (2008)		
Liver (hepatocytes; AFP-Cre)		Hepatocyte differentiation defect	Dai <i>et al</i> (2006); El-Aouini <i>et al</i> (2006); Kanasaki <i>et al</i> (2008)		
Kidney (podocytes; podocin-Cre)	Fibrosis and proteinuria	Belvindrah <i>et al</i> (2006); Mills <i>et al</i> (2006)			
PINCH-1	<i>Caenorhabditis elegans</i> <sup>a</sup> <i>Drosophila melanogaster</i> <sup>a</sup>		Granule cell precursor proliferation defects and Bergmann glial cell differentiation defects	Niewmierzycka <i>et al</i> (2005)	
			Cortical lamination defects	Pereira <i>et al</i> (2009)	
	<i>Mus musculus</i>	Central nervous system (neuroepithelium; Emx1-Cre)	Abnormal radial sorting and remyelination of axons	Akhtar <i>et al</i> (2009)	
		Peripheral nervous system (Schwann cells; Dhh-Cre)	Defects in post-pregnancy mammary gland development and differentiation	Hobert <i>et al</i> (1999)	
	<i>Mus musculus</i>	Mammary gland (mammary epithelial cells; Blg-Cre and Wap-Cre)	Embryonic lethality; muscle attachment defect	Clark <i>et al</i> (2003); Kadrmas <i>et al</i> (2004)	
		Constitutive	Embryonic lethality; actin detachment from muscle membrane, adult wing blisters	Li <i>et al</i> (2005); Liang <i>et al</i> (2005)	
	<i>Mus musculus</i>	Cardiac muscle (ventricular cardiomyocytes; MLC2v-Cre)	Embryonic lethality between E6.5 and E7.5; abnormal epiblast polarization, impaired cavitation, cell detachment from the ECM, abnormal cell-cell contacts, impaired endoderm survival	Liang <i>et al</i> (2005)	
		Constitutive	Viable	Stanchi <i>et al</i> (2005) Liang <i>et al</i> (2009)	
	PINCH-2 and PINCH-2	<i>Mus musculus</i>	Constitutive	Early postnatal lethality; dilated cardiomyopathy	
		<i>Mus musculus</i>	Skeletal and cardiac muscle (cTNT-Cre)	Viable	
	α-Parvin	<i>Caenorhabditis elegans</i> <sup>b</sup> <i>Mus musculus</i>	Constitutive	Embryonic lethality; muscle attachment defect	Lin <i>et al</i> (2003)
		<i>Mus musculus</i>	Constitutive	Embryonic lethality between E10.5 and E14.5; cardiovascular and kidney defects	Lange <i>et al</i> (2009); Montanez <i>et al</i> (2009)
β-Parvin γ-Parvin	<i>Mus musculus</i>	Constitutive	Viable	Thiessen and Fässler (unpublished)	
	<i>Mus musculus</i>	Constitutive	Viable	Chu <i>et al</i> (2006)	

Abbreviations: AFP, α-feto protein; Blg, bovine β-lactoglobulin; Col2, collagen-2; cTNT, cardiac troponin T; Dhh, desert hedgehog homolog; E, embryonic day; EC, endothelial cell; ECM, extracellular matrix; Emx1, empty spiracles homeobox 1; HSA, human skeletal α-actin; K5, keratin 5; K14, keratin 14; Lck, lymphocyte protein tyrosine kinase; Mck, muscle creatine kinase; MLC2v, myosin light chain 2v; Mx1, myxovirus resistant protein 1; PDGFRβ, platelet-derived growth factor receptor β; vSMC, vascular smooth muscle cell; Wap, whey acidic protein.

<sup>a</sup>Possess only a single PINCH isoform.  
<sup>b</sup>Possess only a single parvin isoform.

2008a). Recent studies suggest that ILK does not only regulate the actin cytoskeleton but can also modulate the microtubule network and influence mitotic spindle orientation (Dobrev *et al*, 2008; Fielding *et al*, 2008). However, as loss of ILK can lead to both increased or decreased proliferation rates *in vivo*, depending on the cellular context (Grashoff *et al*, 2003; Sakai *et al*, 2003; Lorenz *et al*, 2007; Gkretsi *et al*, 2008), the relevance of these functions needs to be established.

PINCH-1 is ubiquitously expressed throughout mammalian development and adult life, whereas PINCH-2 expression is observed during the second half of embryonic development and has a slightly more restricted expression pattern (Braun *et al*, 2003). Ablation of PINCH-2 does not affect mouse development, but loss of PINCH-1 results in abnormal epiblast polarity, impaired cavitation, and detachment of endoderm and epiblast from basement membranes (Li *et al*, 2005). However, the functions of PINCH-1 are not restricted to the regulation of cell-matrix adhesions as PINCH-1 has been shown to regulate cell-cell adhesion of the endoderm and epiblast as well as cell survival in the endoderm layer (Li *et al*, 2005). As ILK has not been shown to have a role in these processes, it seems that several functions of PINCH are independent of the IPP complex. How PINCH regulates cell-cell adhesion is not understood, but the mechanisms by which it regulates cell survival are beginning to unravel. In *Drosophila*, PINCH antagonizes the activation of c-Jun N-terminal kinase (JNK) during dorsal closure and seems to fine tune JNK signalling and mediates its cross talk with integrin signalling to allow epithelial morphogenesis. This occurs through binding of PINCH to Ras suppressor 1 (RSU-1), a negative regulator of JNK signalling (Kadmas *et al*, 2004). Interestingly, PINCH-2 cannot bind RSU-1, which might partly explain why it is unable to compensate for the loss of PINCH-1 in mammals (Kadmas *et al*, 2004; Dougherty *et al*, 2005). PINCH-1 is found frequently to be upregulated in human tumours (Eke *et al*, manuscript submitted), and depletion of PINCH-1 results in reduced phosphorylation of Akt on both Ser473 and Thr308 accompanied by decreased cell survival (Eke *et al*, manuscript submitted; Fukuda *et al*, 2003). In addition, PINCH-1 can protect cancer cells from apoptosis through regulation of the Erk-Bim pathway (Chen *et al*, 2008). As Akt activation has been shown to suppress JNK, which in turn downregulates Erk (Junttila *et al*, 2008; Kim *et al*, 2009), it is possible that PINCH-1 regulates all of these pathways by modulating Akt phosphorylation. In line with this possibility is a recent report showing that PINCH-1 binds and inhibits the protein phosphatase 1 $\alpha$  (PP1 $\alpha$ ) (Eke *et al*, manuscript submitted), which can directly dephosphorylate Akt. The inhibition of PP1 $\alpha$  activity leads to increased levels of Akt phosphorylation and increased cell survival upon radiation. Consequently, deletion of PINCH-1 revokes PP1 $\alpha$  inhibition, decreased Akt phosphorylation, and compromised cell survival (Eke *et al*, manuscript submitted).

Like ILK and PINCH, parvins have a role in modulating cell spreading and actin organization downstream of integrins. However, the role of parvins in these processes is more complex and the precise functions of the different isoforms *in vivo* are not clear. Mice lacking  $\beta$ - or  $\gamma$ -parvin show no obvious phenotypes, whereas  $\alpha$ -parvin-null mice die between E11.5 and E14.5, suggesting that the parvin isoforms can functionally substitute for each other during development (Chu *et al*, 2006; Montanez *et al*, 2009; Thievensen and

Fässler, in preparation). All parvins contain two CH domains and bind F-actin *in vitro*, but the functional significance of this interaction is unknown (Oliski *et al*, 2001; Yamaji *et al*, 2001, 2004). The primary sequences of both CH domains of  $\alpha$ -parvin are highly diverged from the typical CH domains found in actin-binding domains (Gimona *et al*, 2002), and it has been shown that  $\alpha$ -parvin uses these domains to interact with paxillin (Nikolopoulos and Turner, 2000; Lorenz *et al*, 2008; Wang *et al*, 2008b). As the C-terminal region containing the CH domains is highly conserved throughout the parvin family, it has been suggested that all parvin paralogues may be able to bind paxillin and its homologue Hic-5 (Lorenz *et al*, 2008). Interestingly, however,  $\gamma$ -parvin can interact with paxillin (Yoshimi *et al*, 2006), to which  $\beta$ -parvin seems not to bind (Yamaji *et al*, 2001), despite being more homologous to  $\alpha$ -parvin than  $\gamma$ -parvin is. In contrast,  $\beta$ -parvin binds the actin-modulatory protein  $\alpha$ -actinin (Yamaji *et al*, 2004). The differential interactions might allow distinct functions for the individual isoforms. Indeed, knockdown of  $\beta$ -parvin in HeLa cells results in reduced cell spreading, whereas depletion of  $\alpha$ -parvin increases cell spreading (Fukuda *et al*, 2003; Yamaji *et al*, 2004). However, most of the cellular studies have been carried out with a single parvin isoform instead of directly comparing the properties of the three proteins. Therefore, additional studies are required to understand the precise functions and signalling specificities of the parvin isoforms.

The use of lower eukaryotes, such as *C. elegans* and *D. melanogaster* as model organisms, has allowed functional analysis of the IPP complex in a simplified system, as they express only a few integrin subunits and single orthologues for PINCH and parvin. Studies in *C. elegans* have shown that ILK (PAT-4), PINCH (UNC-97), and parvin (PAT-6) co-localize with  $\beta$  integrin (PAT-3) at muscle attachment sites termed dense bodies and M-lines, which attach actin filaments to the basal sarcolemma. Deletion of  $\beta$  integrin or any member of the IPP complex leads to a paralysed, arrested elongation at twofold (Pat) phenotype, characterized by embryonic lethality and detachment of muscles from the body wall because of defects in dense body and M-line assembly (Mackinnon *et al*, 2002; Lin *et al*, 2003; Norman *et al*, 2007). In *Drosophila*, PINCH and ILK co-localize with  $\beta$ PS integrins at muscle attachment sites and the basal junctions of the wing epithelium. Similar to the deletion of  $\beta$ PS, both ILK- and PINCH-null flies show detachment of muscles from the body wall (Zervas *et al*, 2001; Clark *et al*, 2003). A parvin mutant has not been described so far. Although in the integrin mutant the detachment occurs between the ECM and the plasma membrane, the ILK- and PINCH-null phenotypes are characterized by detachment of the actin filaments from the muscle ends, establishing an essential function for these proteins in reinforcing the link between integrins and the actin cytoskeleton (Zervas *et al*, 2001; Clark *et al*, 2003). However, loss of integrin function causes additional defects in midgut morphogenesis and dorsal closure (Newman and Wright, 1981; Roote and Zusman, 1995), which are present in the PINCH-null flies (Kadmas *et al*, 2004), but not in the ILK mutants (Zervas *et al*, 2001). This suggests that ILK is indispensable for only a subset of integrin functions and that ILK and PINCH have independent functions in mediating integrin signalling in the fly. In addition, IPP proteins can localize to adhesion sites independently of each other both in *Drosophila* and

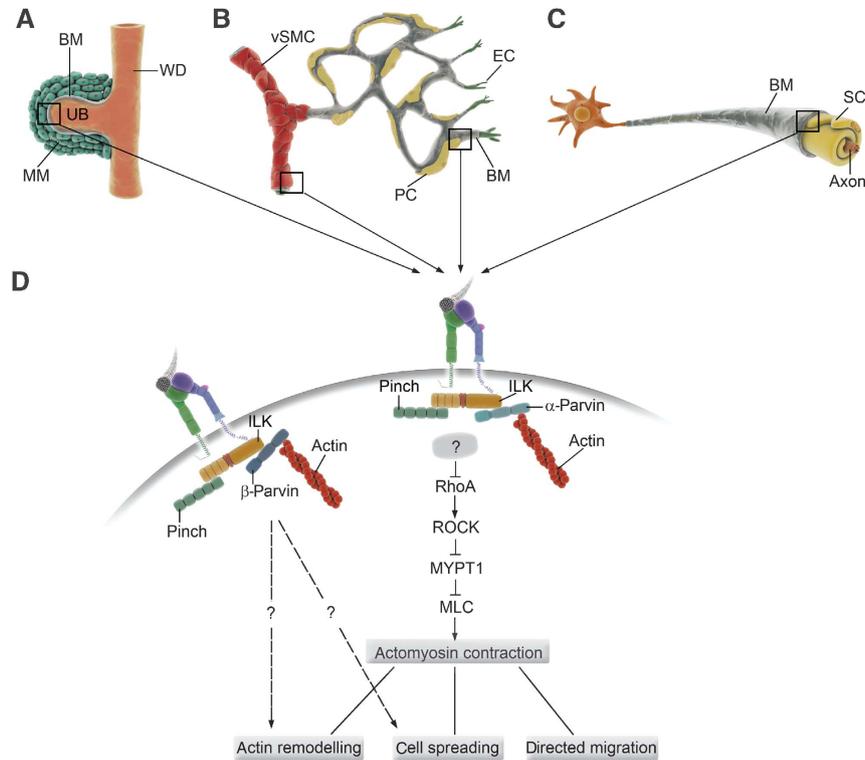
*C. elegans* (Zhang *et al*, 2002; Clark *et al*, 2003; Lin *et al*, 2003). Thus, despite extensive similarities in its role as an essential link between integrins and the actin cytoskeleton, the molecular regulation of the IPP complex seems not to be conserved among species, which might further reflect differences in its function between invertebrates and vertebrates.

### ILK/ $\alpha$ -parvin complex: a negative regulator of cell contractility

Genetic studies have firmly established a role for the IPP complex in adhesion strengthening and organization of the actin cytoskeleton downstream of integrins. However, as several of the functions in mice seem to be tissue specific, the key question as to how this specificity is achieved remains unanswered. The analysis of  $\alpha$ -parvin-null mice has revealed a specific function for this protein in regulating cell contractility in a subset of cell types. Unlike ablation of *ILK* or *PINCH-1*, disruption of the  $\alpha$ -parvin gene in mice does not lead to peri-implantation lethality. Despite the ubiquitous expression pattern of  $\alpha$ -parvin, these mice survive to E14.5, because of the ability of  $\beta$ -parvin to compensate during early development (Montanez and Fässler, unpublished data), and die as a result of severe cardiovascular defects (Montanez *et al*, 2009). The absence of  $\alpha$ -parvin causes impaired investment of vSMCs to developing vessel walls resulting in defective stabilization of the vasculature and subsequent dilation of vessels, formation of microaneurysms, and vessel rupture. These defects are caused by increased RhoA activity that leads to elevated MLC phosphorylation and aberrant actomyosin contractility (Montanez *et al*, 2009). RhoA is a small GTPase that regulates cell contractility through activation of its downstream target ROCK, which in turn can either indirectly activate MLC through phosphorylation and inactivation of MLC phosphatase, or by directly phosphorylating MLC (Amano *et al*, 1996; Kimura *et al*, 1996). Spatiotemporal regulation of RhoA activity is critical for directed cell motility, as activation of RhoA is necessary for the retraction of the trailing edge (BurrIDGE and Wennerberg, 2004), whereas suppression of RhoA activity is required to promote lamellipodial protrusion at the leading edge (Arthur *et al*, 2000; Arthur and BurrIDGE, 2001). On the other hand, RhoA/ROCK signalling has been shown to drive fast, random motility, whereas activation of the GTPase Rac promotes persistent cell migration (Sahai and Marshall, 2003; Danen *et al*, 2005; Sanz-Moreno *et al*, 2008). Consistently with this, the  $\alpha$ -parvin-null vSMCs are hypercontractile and fail to establish a persistent leading edge, resulting in an increase of random motility but a loss of directed migration towards the vessel wall (Montanez *et al*, 2009). Interestingly,  $\alpha$ -parvin-null fibroblasts or endothelial cells do not display a hypercontractile phenotype, suggesting that the function of  $\alpha$ -parvin as a negative regulator of RhoA is cell-type specific (Montanez *et al*, 2009). Tissue-specific ablation of ILK in vSMCs leads to a similar phenotype with impaired coverage of the vasculature by mural cells (Kogata *et al*, 2009). These mice die around E18.5 because of haemorrhages and oedema caused by failure of the vSMCs to form a unitary, stabilizing cell layer around the endothelial tubes, leading to local vessel constriction and rupture. Like in the  $\alpha$ -parvin-null mice, these defects are due to an aberrant upregulation of RhoA/ROCK signal-

ling, resulting in hypercontractility of the vSMCs (Kogata *et al*, 2009). A similar role for ILK in the regulation of Rho activity has been observed in Schwann cells of the nervous system, where ablation of ILK leads to upregulation of Rho/ROCK signalling, resulting in the inability of the Schwann cells to extend cytoplasmic processes to envelope the nerves (Pereira *et al*, 2009). Interestingly, point mutations in the potential ATP-binding site of ILK, which selectively disrupt its interaction with  $\alpha$ -parvin, induce contractile cell behaviour as well as enhanced random motility and loss of directional cell migration in collecting duct epithelial cells (Lange *et al*, 2009), suggesting involvement of a similar RhoA/ROCK-dependent mechanism. These studies collectively identify the ILK/ $\alpha$ -parvin complex as a negative regulator of cell contractility in certain cell types, and point to  $\alpha$ -parvin as the critical modulator of this function. They further suggest that a single parvin isoform seems to be sufficient to fulfil the functions of the IPP complex during early embryonic development of mammals or in less developed organisms. However, later on when differentiation of tissues requires more specialized signalling, such as tight regulation of contractility in the vSMCs or precise spatiotemporal regulation of cell migration in the ureteric bud epithelium, different parvin isoforms construct functionally distinct IPP complexes (Figure 2).

The precise molecular mechanism, by which the ILK/ $\alpha$ -parvin complex regulates RhoA/ROCK signalling, remains open for future research. Interestingly, deleting the  $\beta$ 1 integrin gene in vSMCs also leads to decreased stability of the vasculature (Abraham *et al*, 2008), but the mechanism underlying this defect seems to differ from that of the ILK- and  $\alpha$ -parvin-null mice. Loss of  $\beta$ 1 integrin leads to defective differentiation and aberrant proliferation of vSMC (Abraham *et al*, 2008), but these cells do not display a hypercontractile phenotype or increased levels of MLC phosphorylation indicative of enhanced RhoA activity (Kogata *et al*, 2009). This is in agreement with *in vitro* studies in which the fibronectin receptor  $\alpha$ 5 $\beta$ 1 has been shown to promote RhoA/ROCK signalling, resulting in upregulation of random motility, whereas the fibronectin receptor  $\alpha$ v $\beta$ 3 has been shown to promote directional motility through activation of Rac (Danen *et al*, 2002, 2005). The downstream signals induced by these two heterodimers are regulated by an elegant mechanism of cross talk, where ligation of  $\alpha$ v $\beta$ 3 integrin inhibits the recycling of  $\alpha$ 5 $\beta$ 1 back to the cell surface, resulting in decreased ability of this integrin to promote RhoA/ROCK-mediated random motility (White *et al*, 2007). Conversely, inhibition or downregulation of  $\alpha$ v $\beta$ 3 leads to increased recycling of  $\alpha$ 5 $\beta$ 1 to restore ROCK signalling (White *et al*, 2007). Hence, there are at least two potential scenarios whereby the ILK/ $\alpha$ -parvin complex could operate. In the first scenario, the cell-type specificity of the complex is determined by the integrin heterodimer to which the complex is bound. In vSMCs, the ILK/ $\alpha$ -parvin complex could specifically regulate signalling downstream of  $\beta$ 3 integrin, and ablation of the complex would lead to compromised  $\beta$ 3 signalling and subsequent predominance of  $\beta$ 1 integrin-mediated RhoA/ROCK activation resulting in hypercontractility and loss of persistent cell migration. In the alternative model, the ILK/ $\alpha$ -parvin complex could function as a negative feedback loop for  $\beta$ 1 integrin through recruitment of negative regulator(s) of the RhoA/ROCK/MLC2 signalling



**Figure 2** Model of how the ILK/ $\alpha$ -parvin complex regulates tissue morphogenesis by suppressing cell contractility. (A–C) Examples of morphogenetic events that require tight regulation of cell contractility for promoting directional migration and cellular organization. (A) Kidney development is initiated by the outgrowth of the ureteric bud (UB) from the Wolffian duct (WD) to invade the surrounding metanephric mesenchyme (MM) resulting in nephron formation and formation of the collecting duct system. BM, basement membrane. (B) Vascular smooth muscle cells (vSMC) and pericytes (PC) are recruited by the endothelial cells (EC) to surround large arteries and capillaries, respectively. The recruited vSMCs and PCs subsequently spread around the vessels to stabilize the endothelial tubes, to guide vascular remodelling, and to regulate vessel tone. (C) Schwann cells extend cellular processes and wrap axons of neurons to generate a myelin sheet that promotes neuronal survival and enhances the conduction velocity of nerve impulses. (D) Two molecularly distinct IPP complexes associate with integrins at cell-matrix adhesions. The ILK/PINCH/ $\alpha$ -parvin complex functions as a mechanosensor to downregulate RhoA signalling downstream of cell adhesion, possibly by recruiting an unidentified negative regulator of RhoA (illustrated with a boxed question mark). Suppression of RhoA activity leads to decreased activation of the downstream target ROCK. ROCK regulates contractility by inactivating the myosin light chain phosphatase (MYPT), which dephosphorylates and inactivates myosin light chain (MLC). ROCK has also been shown to directly phosphorylate and activate MLC. Activated MLC promotes actomyosin contractility. Spatiotemporal regulation of RhoA activity and actomyosin contractility is essential to promote actin remodelling, cell spreading, and directed cell migration. Enhanced RhoA activity, which occurs in the absence of ILK/ $\alpha$ -parvin, leads to actomyosin hypercontractility resulting in enhanced actin stress fibre formation, decreased cell spreading, and loss of directional migration. The specific functions of the ILK/PINCH/ $\beta$ -parvin complex are not clear, but are likely to involve remodelling of the actin cytoskeleton downstream of integrin adhesion.

pathway to this complex. The tissue specificity of the signalling would then be achieved by the relative expression levels of  $\alpha$ -/ $\beta$ -parvin and/or by the cell-type-specific expression pattern of the yet unidentified negative regulator(s) downstream of  $\alpha$ -parvin (Figure 2).

## Concluding remarks

Recent studies have led to important and exciting advances in the overall as well as specific understanding of IPP complex functions. It is now clear that the putative kinase activity of ILK is non-existent and thus cannot be required for its function *in vivo*, and that the kinase homology domain is a critical mediator of several protein–protein interactions. Therefore, ILK is an essential scaffold protein, whose central function is to target the IPP complex to integrin adhesion sites. Whether this involves a direct interaction of ILK with  $\beta$  integrins and how kindlin and paxillin cooperate to modulate the recruitment of the complex to FAs require more analyses. Comparative studies using different cell types might further

reveal specific modes of recruitment that could enable tissue-specific functions of the IPP complex as well as its individual components.

The assembly of molecularly distinct IPP complexes has turned out to be another critical determinant of tissue specificity, but the mechanisms that regulate the relative abundance of the various complexes, as well as the distinct functional properties of the individual components that ultimately generate this specificity remain unknown. Identification of accessory proteins that cooperate with the IPP complex to regulate tissue-specific functions as well as biochemical and structural studies comparing the different PINCH and parvin isoforms are needed to understand the molecular determinants for the specific functions of these complexes. Finally, as most of the *in vivo* studies on the IPP complex proteins have been carried out using constitutive or tissue-specific deletions of the components, which leads to a significant reduction in the levels of the other members, further *in vivo* analyses using point mutations that specifically disrupt the various interactions of these proteins will be

an important approach to overcome this limitation and to provide more insights into the functions of these proteins in development and disease.

### Note added in proof

After submission of our manuscript Qin and co-workers (Fukuda *et al*, 2009) reported the high-resolution crystal structure of the ILK kinase domain, which revealed a kinase fold with a distinct pseudosubstrate active site conformation, further corroborating ILK as a pseudokinase.

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## Conflict of interest

The authors declare that they have no conflict of interest.

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